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Hakija Applicant	1. Laukkanen, Mikko, Kuopio 2. Ylä-Herttuala, Seppo, Vuorela
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Osoite: Arkadiankatu 6 A, P.O.Box 1160
Puhelin: 09 6939 500, Telephone: + 358 9 6939 500
Telefax: 09 6939 5328, Telefax: + 358 9 6939 5328

Extracellular superoxide dismutase (EC-SOD) gene therapy for inhibiting restenosis

FIELD OF THE INVENTION

The invention relates to the use of adenovirus-mediated extracellular superoxide dismutase (EC-SOD) gene/cDNA transfer to aorta and other blood vessels on neointima formation.

BACKGROUND OF THE INVENTION

Conventional treatment of atherosclerotic vascular diseases has improved significantly due to the use of drugs, angioplasty, bypass grafting, endartherectomy, and stent implantation. However, restenosis is still an important complication of the invasive treatment. By restenosis it is meant the reoccurrence of stenosis, which is a condition result of disease in which the aorta and especially its orifice is abnormally narrow, in a blood vessel after it has been treated (as by angioplasty) with apparent success. It occurs in 20-30% of patients and is due to proliferation and migration of smooth muscle cells and myofibroblasts, which leads to formation of neointima. By neointima it is meant a new or thickened layer of arterial inner layer of blood vessel formed by migration and proliferation of cells from the media.

Restenosis is a vascular response to percutaneous transluminal coronary angioplasty (PTCA), coronary artery bypass grafting (CABG) and coronary stenting causing intimal hyperplasia formation in 20-30% of patients1; 2; 2.Balloon angioplasty frequently causes intimal tearing, endothelial cell damage and exposure of subendothelial tissue to blood components3 leading to increased oxidative stress, superoxide anion (O2-) production4; 5; 5,{Laurindo, da Luz, et al. 1991 ID: LAURINDO1991} and decreased vascular superoxide dismutase concentration4.

Treatment of restenosis has so far been largely unsuccessful despite of numerous efforts. Restenosis is most effectively inhibited by inducing endothelial cell growth with vascular endothelial growth factors (VEGF) and nitric oxide (NO) related genes6. VEGF-A or VEGF-C induce endothelial repair but do not decrease oxidative stress and infiltration of macrophages. Macrophages are known to secrete many cytokines and growth factors which induce SMC proliferation and participate neointima formation7; 8.

In the US patents 5,788,961 and 5,472,691 the EC-SOD protein has been studied for its therapeutic effect in treating ischemia and inflammatory diseases and other conditions of the body associated with the presence or formation of superoxide radicals. However, gene therapy by the EC-SOD gene/cDNA has not been evaluated.

SUMMARY OF THE INVENTION

The object of the invention is to analyse the effects of adenovirus-mediated extracellular superoxide dismutase (EC-SOD) gene/cDNA transfer to aorta and other blood vessels on neointima formation.

Secreted EC-SOD as an antioxidative9 enzyme may offer a potential tool for vascular gene/cDNA therapy since it is capable of binding to heparan sulphate proteoglycans on the surface of vascular endothelial cells10. In previous studies EC-SOD has been shown to reduce O2- mediated macromolecular and cellular damages in vitro and in vivo suggesting that EC-SOD gene/cDNA therapy may be used to correct tissue damages caused by free radicals9; 11; 11; 12; 12; 13; 13. In addition to dismutase activity EC-SOD, and other SODs, have been hypothesised to have other enzymatic properties, which would enlarge the biological range of the SOD family14.

About 50% of the total SOD activity in human aorta is EC-SOD15; 16; 16. In most of the other tissues EC-SOD represents only a minor part of the total SOD activity17, which suggests that EC-SOD has a significant physiological role in redox balance of vascular walls.

The applicant has shown that adenovirus mediated EC-SOD gene/cDNA transfer resulted in a significant ($p<0.001$) inhibition of neointima formation in rabbit aortas after balloon denudation. It was found that EC-SOD inhibited significantly neointima formation. The therapeutical effect was affecting the whole abdominal aorta studied suggesting a systemic effect. According to the present application EC-SOD is an efficient therapeutical molecule to prevent restenosis.

BRIEF DESCRIPTION OF THE FIGURES

The invention will now be described in detail with reference to the figure.

Figure 1. Histological analysis of serial sections from abdominal aorta two weeks after gene/cDNA transfer. Panels A, C, E, G, I, K, and M represent EC-SOD group

and panels B, D, F, H, J, L, and N represent LacZ group. Arrows indicate the location of internal elastic lamina. A. and B. H-E staining; C and D internal elastic lamina determination; E and F endothelium staining (CD-31); G and H BrdU staining; I and J macrophage staining (Ram 11); K and L NADPH-oxidase staining (p67phox); M and N SMC staining (HHF-35). Intima/media area ratio, cell proliferation, and accumulation of macrophages were significantly decreased in EC-SOD group whereas endothelium recovery was significantly increased in EC-SOD group as compared to LacZ controls. Measurement of internal elastic lamina showed similar damage in both groups.

DETAILED DESCRIPTION OF THE INVENTION

Oxidative stress is associated with several diseases and tissue damage either as a cause or a consequence of a disease. Increased production of reactive oxygen species is detected in vascular disorders, such as ischemia and atherogenesis leading to oxidative stress. Oxidative stress is attenuated by antioxidative agents and several types of antioxidative enzymes.

Extracellular superoxide dismutase (EC-SOD) is a secreted antioxidative enzyme, which is widely expressed throughout the body and is the major SOD isoenzyme in plasma. Vessel wall, lung, kidney, thyroid gland, and epidymis are shown to be the primary expression sites for the EC-SOD. About 50% of total SOD amount in human aorta is EC-SOD. In most other tissues EC-SOD represents only a minor part of the total SOD activity, which suggests that EC-SOD has a significant physiological role in redox balance of vascular wall.

In the present application rabbit extracellular superoxide dismutase (EC-SOD), which is one of the three superoxide dismutase isoforms known in mammals, is used to study the adequacy of EC-SOD for gene therapy. The rabbit cDNA and gene were cloned from phage libraries for sequence, mutation, and expression analysis9; 18. The expression and functionality of EC-SOD was studied in vitro and in vivo using nonviral and adenovirus mediated gene/cDNA transfer methods. Therapeutical effects were tested in mouse and rabbit models11; 19.

The antioxidative role and therapeutical properties were shown in vitro and in vivo. EC-SOD isolated from culture medium after adenovirus mediated gene/cDNA transfer reduced endothelial cell-mediated oxidative modification of low density lipoprotein (LDL) suggesting the functionality of the cloned cDNA9. The therapeutical properties were tested in mice11; 19 and rabbits after adenovirus

mediated gene/cDNA transfer. In mice EC-SOD efficiently inhibited paracetamol-induced liver damage and in New Zeeland White (NZW) rabbits EC-SOD efficiently attenuated neointima formation after balloon denudation, indicating a broad therapeutic window and functionality of EC-SOD in different radical based dysfunctions. Since oxygen free radicals are involved in the formation of tissue injury, EC-SOD could be useful for therapeutical applications in several diseases.

There are only a few gene/cDNA therapy studies with EC-SOD11; 11-13; 13 even though EC-SOD is potentially more suitable for vascular mediated gene/cDNA transfer than other SODs. It is a secreted enzyme20 capable to bind reversibly to heparan sulphate on the cell membranes10, and has a remarkable long (20 h) half life in circulation21. Recent studies with EC-SOD transgenic animals have shown that EC-SOD can significantly reduce tissue damage caused by oxygen derived free radicals22; 23; 23; 24; 24; 25; 25; 26; 26; 27; 27.

In the present application it is shown that adenovirus mediated EC-SOD gene/cDNA transfer resulted in a significant ($p<0.001$) inhibition of neointima formation in ballooned rabbit aortas. EC-SOD transduced rabbits had significantly increased endothelium recovery and significantly reduced macrophage accumulation into vessel wall. Both of these alone have been reported to reduce neointima growth in animal models8; 28. The reduced infiltration of macrophages suggests anti-inflammatory role for EC-SOD in addition to antioxidative role which have been shown in previous studies9. Results from BrdU labelling showed low proliferation in EC-SOD transduced vessels.

In the present application it is analyzed the effects of adenovirus-mediated extracellular superoxide dismutase (EC-SOD) gene/cDNA transfer on neointima formation after endothelial denudation in rabbits. Two weeks after the gene/cDNA transfers (3x109 pfu/kg AdEC-SOD or AdLacZ) aortas were isolated and neointima formation was studied using histological and morphometric analysis. Intima/media ratio in EC-SOD transduced rabbits was significantly ($p<0.001$) decreased in the whole abdominal aorta as compared to LacZ transduced control rabbit abdominal aortas suggesting a systemic effect. The recovery of endothelial cells was significantly ($p<0.001$) increased whereas the number of macrophages ($p<0.001$), and BrdU positive cells were significantly reduced in EC-SOD rabbit aortas (intima $p<0.01$ and media $p<0.05$). The results suggest that delivery of EC-SOD adenoviruses is a useful tool for the treatment of postangioplasty restenosis and vessel wall thickening after vascular manipulations.

The invention will be further described with reference to the following non-limiting examples.

Example 1

Animal experiments

20 New Zeeland White rabbits (EC-SOD n=10 and LacZ n=10) were kept on 0.25% cholesterol diet for two weeks before balloon denudation. In the denudation the whole aorta beginning from the tip of the arch was denuded using 3.0 F arterial embolectomia catheter (Sorin Biomedical, Irvine, CA). The catheter was introduced via the right iliac artery up to the aortic arch and inflated, and the aorta was denuded twice29.

Three days after denudation adenovirus mediated EC-SOD or LacZ control gene/cDNA transfer (2x109 pfu) was performed using systemic virus injection or Dispatch catheter (Boston Scientific Corp., Maple Grove, MA) which allows continuous blood flow during transduction30. Using fluoroscopical control, the catheter was positioned caudal to the left renal artery via 4 F percutaneous introducer sheath (Arrow International, Reading, PA) in the right carotid artery inflated to 6 ATM with the mixture of contrast media and saline. The gene transfer was performed for 10 minutes (0.2 ml/min. total volume being 2 ml). Two weeks after gene/cDNA transfer adenovirus transduced aortas were isolated. Serum samples were collected before gene/cDNA transfer, three days after the gene/cDNA transfer, seven days after the gene/cDNA transfer and at the end of the experiment. Tissue samples were collected to determine the distribution of adenovirus.

Example 2

Adenovirus production

The EC-SOD cDNA was cloned into BamHI site of eukaryotic expression plasmid31, which contains elongation factor 1α (EF1α) promoter. The resulting expression cassette contains EF1α promoter followed by rabbit EC-SOD cDNA followed by G-CSF polyadenylation signal. The EC-SOD expression cassette was cloned into an adenovirus vector (AdBglII) by digesting eukaryotic expression vector with PvuII restriction enzyme. The vector AdBglII was digested with BglII and the overhangs were filled. EC-SOD expression cassette was subcloned into AdBglII with blunt-end ligation9. LacZ adenovirus was used as a control32. Adenoviruses were produced in 293 cells and analysed to be free of microbiological

contaminants, mycoplasma, endotoxin and replication-competent viruses9. The adenovirus production was done using standard recombination techniques33.

Example 3

EC-SOD activity analysis

The efficiency of adenovirus gene transfer was determined by measuring total SOD activity from the plasma as described34. Briefly, 25-50 µl sample was added to 3 ml 50mM AMP/HCl pH 9.5/0.2 mM DTPA buffer and blanked before addition of kaliumperoxide (KO2) substrate in 50 mM NaOH/0.5 mM DTPA (Sigma). The reaction was followed five minutes at wavelength 250 nm (Lambda Bio, Perkin Elmer). The activity was calculated by determining the half-times of O2- decay at A250. One unit in the assay is defined as the activity that brings about a decay in O2- concentration at a rate of 0.1s-1 in 3ml buffer and corresponds to 8.6 ng of human EC-SOD as quantified by ELISA34.

Example 4

Histological analysis

Four hours before sacrification, animals were injected with 50 mg iv. of bromodeoxyuridine (BrdU) (Sigma) dissolved in 40% ethanol. Transduced segment of the aorta was removed, flushed gently with saline and divided into four equal parts used for histological paraffin sections, cryo sections, BrdU sections and one piece stored at –70 C°. Paraffin sections were immersion-fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for four hours, rinsed overnight in 15% sucrose (pH 7.4) and embedded in paraffin. Cryo sections were fixed in 4% paraformaldehyde/15% sucrose (pH 7.4) for 10 minutes, rinsed in PBS, embedded in O.C.T. compound (Miles Scientific, Elkhart) and stored at –70 C. BrdU sections were fixed in 70% ethanol for five hours and embedded in paraffin. Gene transfer efficiency was determined by x-gal staining in x-gal staining solution for 6 hours at +37 C35. Neointima formation was measured after hematoxylin/eosin staining using Image-pro plus software with Olympus AX70 microscope (Olympus Optical). Determination of BrdU positive cells was done using immunohistochemistry (Bromodeoxiuridine, clone Bu20a, dilution 1:200, DAKO) according to manufacturers intructions.

Following antibodies were used to study the effect of gene transfer: CD31 (endothelium, dilution 1:50, DAKO), RAM 11 (macrophages, dilution 1:200,

DAKO), HHF35 (SMC, dilution 1:50, DAKO), p67phox (NADPH oxidase, dilution 1:100, Transduction Laboratories) eNOS (dilution 1:25, Transduction Laboratories), iNOS (dilution 1:50, Transduction Laboratories), VEGF-A (dilution 1:100, Santa Cruz), VEGF-C (dilution 1:100, Santa Cruz), NF-kappaB (dilution 1:50, Transduction Laboratories), BrdU positive cells (Bromodeoxiuridine, clone Bu20a, dilution 1:200, DAKO). Avidin-biotin-horseradish peroxidase system was used for signal detection (Vector Elite Kit).

Example 5

5.1 Statistical analysis

Statistical analysis was done using ANOVA and modified t-test.

Gene transfer site and adjacent portions of abdominal aorta were analysed immunohistologically from a total of 20 animals (10 EC-SOD and 10 LacZ control rabbits) to study the effect of adenoviral EC-SOD gene transfer on restenosis.

5.2 Histological analysis

To determine the effect of EC-SOD gene/cDNA transfer on neointima formation the intima-media area ratio from aortic samples was measured. Histological analysis showed significantly ($p<0.001$) reduced neointimal hyperplasia in EC-SOD group as compared to LacZ controls in the region from renal artery to aortic bifurcation suggesting systemic effect for prevention of restenosis (Fig. 1 a and b)). The mean (±SD) neointima/media area ratio in EC-SOD transduced rabbits was 0.091 ±0.054 and in LacZ rabbits 0.326 ±0.098 indicating significant ($p<0.001$) reduction in restenosis.

Since the neointima formation in balloon denuded rabbit model is dependent on the endothelial balloon damage the length of internal elastic lamina (IEL) was determined from each aortic sample (Fig. 1 c and d). The measurement showed 4% damage to IEL in both EC-SOD and in LacZ control groups indicating similar damage to endothelium after denudation. Aortic sections stained with CD31 for endothelial cells showed 86% ($p<0.001$) recovery of vessel endothelium after denudation in EC-SOD group whereas in LacZ control group the endothelial recovery was only 21% (Fig. 1 e and f).

BrdU staining (Fig. 1 g and h) showed 10-fold higher proliferation ($p<0.01$) in neointima and 2-fold higher ($p<0.05$) proliferation in media of LacZ control group

as compared to EC-SOD group. Activated macrophages express several cytokines and growth factors, such as interleukin-1, platelet derived growth factor, and insulin-like growth factor 136. RAM 11 staining for macrophages (Fig. 1 i and j) showed a 10-fold decreased ($p<0.001$) macrophage infiltration into neointima in EC-SOD group. NADPH oxidase, which is reported to be up-regulated in restenotic rabbit aortas5, was stained at the similar regions as macrophages (Fig. 1 k and l). Intimal thickening is mostly caused by migration and proliferation of SMCs37 (Fig. 3 m and n) but also macrophages are present in high abundance38.

The results show that EC-SOD gene/cDNA transfer is a useful tool for the treatment of postanglioplasty restenosis and vessel wall thickening after vascular manipulations.

The invention has been illustrated by examples and embodiments, but it may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention and all such modifications are intended to be included within the scope of the enclosed claims.

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Claims

1. Extracellular superoxide dismutase (EC-SOD) gene/cDNA for the treatment of restenosis.

2. Extracellular superoxide dismutase (EC-SOD) gene/cDNA for the treatment of blood vessel thickening.

3. Extracellular superoxide dismutase (EC-SOD) gene/cDNA for increasing endothelial cell growth.

4. Extracellular superoxide dismutase (EC-SOD) gene/cDNA for decreasing macrophage accumulation.

5. Use of extracellular superoxide dismutase (EC-SOD) gene/cDNA for the treatment of restenosis.

6. Use of extracellular superoxide dismutase (EC-SOD) gene/cDNA for the treatment of blood vessel thickening.

7. Use of extracellular superoxide dismutase (EC-SOD) gene/cDNA for increasing endothelial cell growth.

8. Use of extracellular superoxide dismutase (EC-SOD) gene/cDNA for decreasing macrophage accumulation.

9. The use according to claims 5 to 8 wherein adenovirus or plasmid containing EC-SOD is used.

10. The use according to claims 5 to 9 wherein rabbit EC-SOD cDNA is used.

11. Use of extracellular superoxide dismutase (EC-SOD) gene/cDNA for manufacturing of a medicament for treating of restenosis.

12. Use of extracellular superoxide dismutase (EC-SOD) gene/cDNA for manufacturing of a medicament for treating of blood vessel thickening.

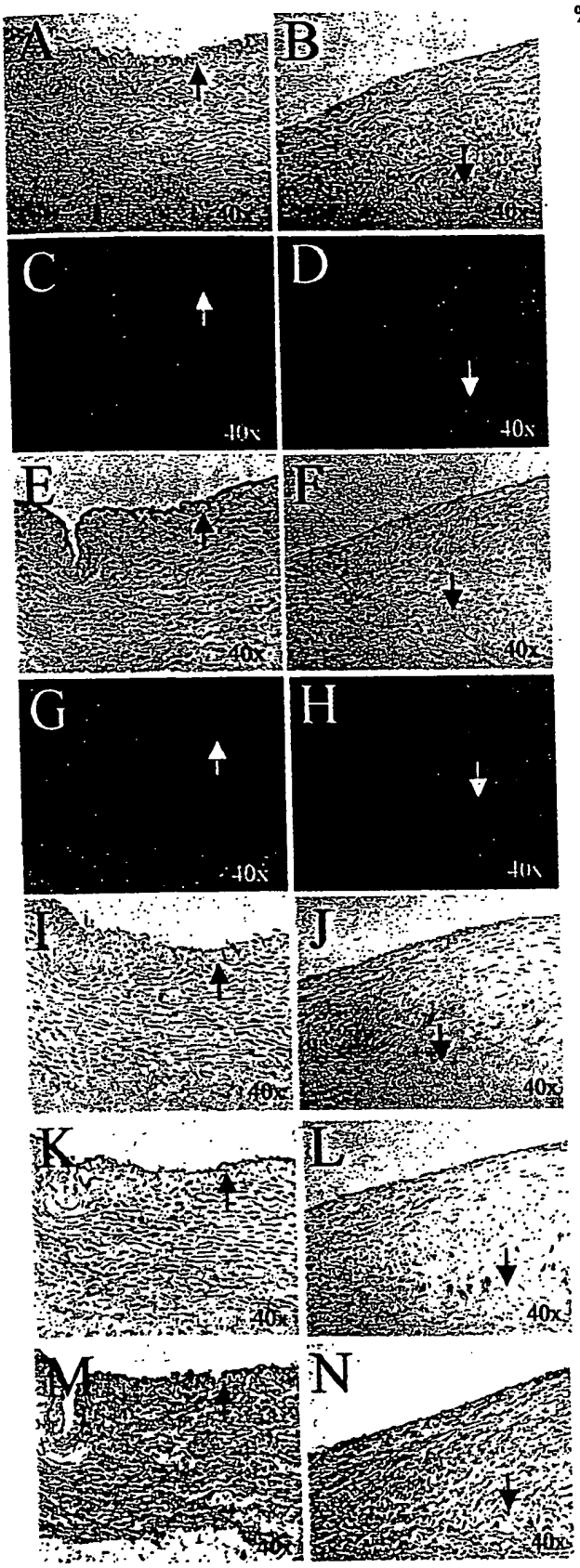
13. Use of extracellular superoxide dismutase (EC-SOD) gene/cDNA for manufacturing of a medicament for increasing endothelial cell growth.

14. Use of extracellular superoxide dismutase (EC-SOD) gene/cDNA for manufacturing of a medicament for decreasing macrophage accumulation.

15. Use of one of the claims 11 to 14 wherein the medicament is administered by local or systemic delivery.

(57) Abstract

The invention relates to the use of adenovirus-mediated extracellular superoxide dismutase (EC-SOD) gene/cDNA transfer to aorta and other blood vessels on neointima formation.



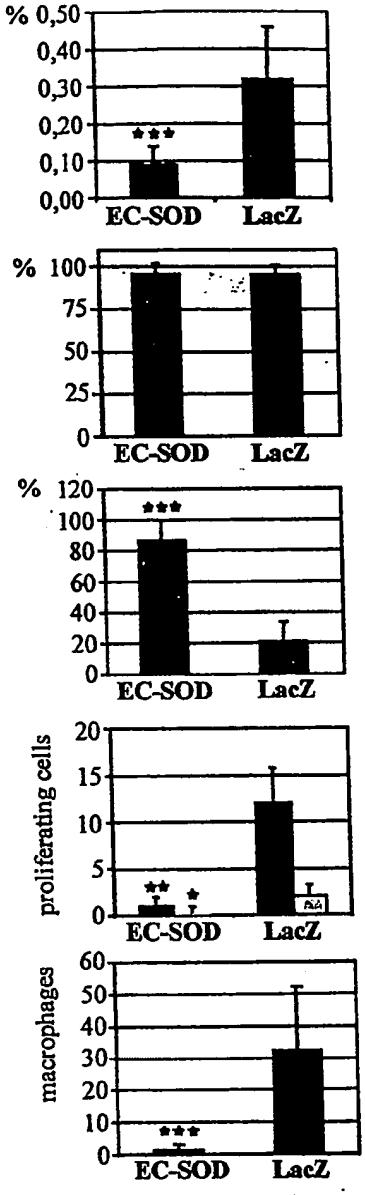


Fig. 1